MINIREVIEW

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Non-redundant Functions of Cyclooxygenases: Oxygenation of Endocannabinoids*

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The two cyclooxygenase (COX) enzymes catalyze the oxygenation of arachidonic acid to prostaglandin endoperoxides, which are the common intermediates in the biosynthesis of the bioactive lipids prostaglandins and thromboxane. COX-1 and COX-2 are ~60% identical in amino acid sequence, exhibit highly homologous three-dimensional structures, and appear functionally similar at the biochemical level. Recent work has uncovered a subtle functional difference between the two enzymes, namely the ability of COX-2 to efficiently utilize neutral derivatives (esters and amides) of arachidonic acid as substrates. Foremost among these neutral substrates are the endocannabinoids 2-arachidonoylglycerol and arachidonoylethanolamide. This raises the possibility that COX-2 oxygenation plays a role in a novel signaling pathway dependent on agonist-induced release of endocannabinoids and their selective oxygenation by COX-2. Among the products of COX-2 oxygenation of endocannabinoids are glyceryl prostaglandins, some of which (*e.g.* glyceryl prostaglandin E_2 and glyceryl prostaglandin I_2) exhibit interesting biological activities in inflammatory, neurological, and vascular systems. These compounds are produced in intact cells stimulated with physiological agonists and have been isolated from *in vivo* sources. Important concepts relevant to the hypothesis of a COX-2-selective signaling pathway are presented.

Cyclooxygenases (COX-1 and COX-2)[2] catalyze the committed step in the conversion of AA to PGs, thromboxane, and PGI_2 and, in so doing, trigger the biosynthesis of an important family of lipid mediators (1, 2). Cyclooxygenase activity was first described in 1964 (3), and COX-1 was purified in 1976 (4). These events occurred concomitantly with the realization that nonsteroidal anti-inflammatory drugs achieve their anti-inflammatory effects primarily by blocking the cyclooxygenase reaction (5). The discovery of COX-2 generated important insights into inflammation, wound healing, reproduction, renal function, and vascular biology *inter alia*, leading to a pharmacological strategy for the treatment of inflammation with reduced gastrointestinal toxicity and providing a new target for the prevention of cancer (6, 7). Despite the rapid pace of these discoveries, our understanding of the physiological roles of the two COX enzymes is incomplete, especially with regard to potential non-redundant functions (8).

Ptgs-1, which codes for COX-1, is transcribed constitutively into a 2.8-kb mRNA, whereas *Ptgs-2* is an immediate-early gene that produces a 4-kb mRNA in response to a wide range of stimuli. COX-1 mRNA is relatively stable, whereas COX-2 mRNA turns over rapidly because of the presence of instability sequences in the 3′-untranslated region. Human COX-1 and COX-2 contain 576 and 580 amino acids, respectively, and are 60% identical in sequence (9–12). The major elements of the primary structures are comparable, so the domain structures are identical, and the three-dimensional structures are essentially superimposable. Both COX enzymes are located in the lumen of the endoplasmic reticulum and in the nuclear envelope (13, 14).

COX-1 and COX-2 catalyze the oxygenation of polyunsaturated fatty acids to hydroperoxy endoperoxides at the cyclooxygenase active site and the reduction of the hydroperoxide to an alcohol at the peroxidase active site (Fig. 1) (15). Each protein uses a free radical mechanism in which an initial reaction with a hydroperoxide generates a higher oxidation state of the heme prosthetic group, which oxidizes an active-site tyrosine to activate the oxygenase (16–18). COX-2 is more sensitive to hydroperoxide-dependent activation compared with COX-1 (~10-fold), which may result in differential activation of the two enzymes in cells with low peroxide concentrations (19). Additional biochemical differences between the COX proteins are those related to the utilization of different polyunsaturated fatty acid substrates (20, 21) and differences in protein turnover (22).

It is possible that the differential transcriptional responses to cell stimuli are the only physiologically relevant distinction between the COX enzymes. However, many of the cells that express COX-2 already express functional COX-1, so the net increase in PG production is only 2–3-fold even following dramatic increases in the levels of COX-2 (23). COX-2 is expressed constitutively in specialized regions of the brain and kidney (24, 25) and may represent the sole source of PGs in those areas. However, this situation is the exception rather than the rule with respect to tissue and cellular localization.

Yu *et al.* (26) recently tested the interchangeability of the two enzymes by knocking *Ptgs-1* into the *Ptgs-2* locus in mice. RPMs from these animals demonstrated inducibility of COX-1 protein in response to LPS treatment but were unable to produce PGs at low concentrations of AA, as anticipated by the differences in hydroperoxide activation described above. The *Ptgs-1* knock-in partially restored the deficit in the major urinary PGI_2

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[2] The abbreviations used are: COX, cyclooxygenase (prostaglandin G/H synthase); AA, arachidonic acid; PG, prostaglandin; PGI_2, prostacyclin; RPMs, resident peritoneal macrophages; LPS, lipopolysaccharide; AEA, arachidonoylethanolamide; 2-AG, 2-arachidonoylglycerol; PG-G, glyceryl prostaglandin; PG-EA, ethanolamide prostaglandin; IP_3, inositol 1,4,5-trisphosphate; ERK, extracellular signal-regulated kinase; mIPSCs, miniature inhibitory postsynaptic currents; EP receptor, E series PG receptor; MAPK, mitogen-activated protein kinase; IL, interleukin; FP receptor, F series PG receptor; PPARδ, peroxisome proliferator-activated receptor-δ; siRNA, small interfering RNA; DAG, diacylglycerol; PL, phospholipase.

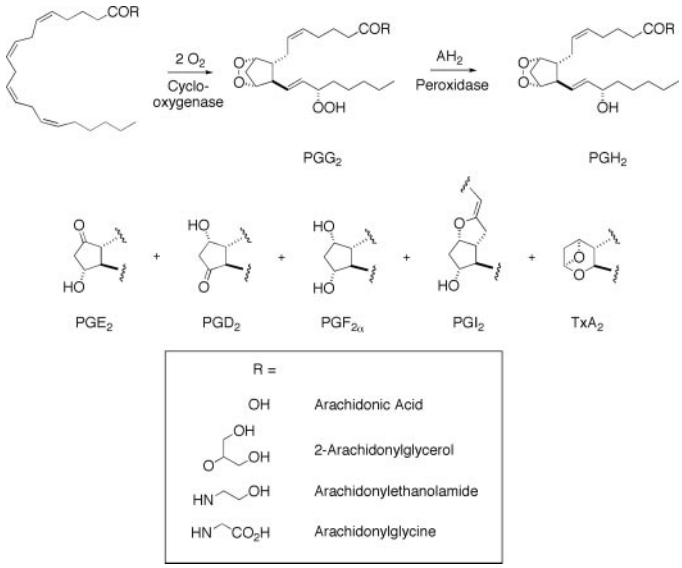

FIGURE 1. **COX-2-dependent oxygenation of AA and its neutral derivatives.** Oxygenation of neutral AA derivatives produces the analogous PG metabolites in every case except for thromboxane A_2 (*TxA_2*).

metabolite observed in *Ptgs-2* knock-out animals, whereas the deficit in the major urinary PGE_2 metabolite was completely restored. This suggests that there may be differences in coupling between the two oxygenases and downstream synthases. Deficiencies in reproductive and renal function observed in *Ptgs-2*-deficient mice were partially corrected or delayed, respectively, in *Ptgs-1* knock-in mice. These mice will serve as an excellent resource with which to probe non-redundant functions of the two COX enzymes.

Selective Oxygenation of Ester and Amide Substrates by COX-2

A major structural difference between COX-1 and COX-2 is the size of their cyclooxygenase active sites (Fig. 2) (27). The presence of a side pocket near the base of the active site of COX-2 makes its site 24% larger than that of COX-1. This side pocket was utilized accidentally in the development of the diarylheterocycle class of COX-2-selective inhibitors, which possess a sulfone or sulfonamide group that inserts into the side pocket of COX-2 (28). Ile-523 in COX-1 acts as a gatekeeper to prevent stable binding of sulfones or sulfonamides in the space corresponding to the side pocket of COX-2. In addition to V523I, other conserved COX-2 to COX-1 substitutions in this region include R513H and V434I.

Although it represents an important motif for pharmacological targeting, the COX-2 side pocket clearly did not evolve for this purpose. Has the side pocket structure been conserved to confer additional functionality on COX-2? Yu *et al.* (29) and Kozak *et al.* (30) demonstrated that COX-2 oxygenates neutral derivatives of AA (*e.g.* AEA and 2-AG) much more efficiently than does COX-1 (~20-fold more). In fact, 2-AG is as good a substrate for COX-2 as is AA, exhibiting comparable k_{cat}/K_m values for both human and mouse COX-2 (30). Site-directed mutagenesis studies show that the ability of COX-2 to use neutral substrates is associated principally with its side pocket and that Arg-513 makes the major contribution to the oxidation of 2-AG and AEA (31).

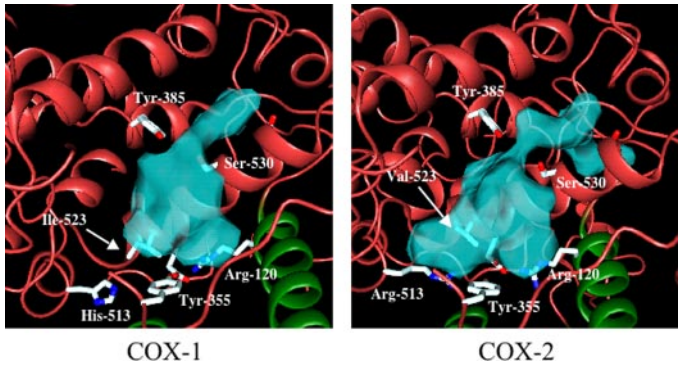

FIGURE 2. **Comparison of the structures of the COX-1 and COX-2 active sites.** The *blue* surface represents solvent-accessible space. The larger space in the COX-2 active site is due to a side pocket that is poorly accessible in COX-1 because of the added steric bulk at position 523 (Ile in COX-1 and Val in COX-2).

Did the conserved side pocket of COX-2 evolve to endow the enzyme with an expanded substrate specificity and thereby a novel function? 2-AG and AEA are members of a family of arachidonoyl derivatives that exist physiologically. 2-AG and AEA are the most extensively studied of this family because they were the first two endogenous ligands described for the cannabinoid receptors (CB_1 and CB_2) (32). 2-AG and AEA are widely distributed in mammalian tissues, although 2-AG is usually present at levels 2–3 orders of magnitude higher. Structure-activity studies of COX-2 oxygenation of 2-AG and AEA indicate that at least one hydroxyl group is required in the ester or amide side chain to render the compound an efficient substrate (33). Among a series of some 30 natural and synthetic arachidonoyl esters and amides that have been tested, 2-AG appears to be the best substrate for COX-2. 2-AG isomerizes to 1-AG with a half-life of 4–10 min under physiological conditions, which reduces COX-2-dependent oxygenation by ~60%. COX-2 oxidizes the arachidonoyl amino acid 2-arachidonoylglycine, but its k_{cat}/K_m is ~10% that of AA (34). The recently reported arachidonoyltaurine is not a substrate nor are arachidonoyl-containing diacylglycerols or arachidonoylcholesterol (35).

Products of COX-2-selective Oxygenation

The products of COX-2 oxygenation of 2-AG, AEA, and arachidonoylglycine are hydroxy endoperoxides analogous to PGH_2 (*i.e.* PGH_2-G, PGH_2-EA, and PGH_2-glycine) (29, 30). PGH_2-G and PGH_2-EA are metabolized by downstream synthases to a similar range of products as PGH_2 (Fig. 1) (36). The one exception is conversion to thromboxane A_2 analogs. PGH_2-G and PGH_2-EA appear to be poor substrates for thromboxane synthase (36).

The neutral PG derivatives are poor substrates compared with the PGs for oxidation by 15-hydroxyprostaglandin dehydrogenase (37). The relative substrate specificities for oxidation of PGE_2, PGE_2-EA, and PGE_2-G are 1:0.36:0.22. $PGF_{2\alpha}$ is a poorer substrate for the dehydrogenase ($V_{max}/K_m = 0.2$) compared with PGE_2, so, perhaps not surprisingly, $PGF_{2\alpha}$-G does not appear to be oxidized at all. These findings suggest that PG-Gs or PG-EAs are able to diffuse farther from the site of their generation compared with classical PGs.

Both PG-Gs and PG-EAs are relatively stable in human serum or plasma. Neither is hydrolyzed in serum, and PG-Gs exhibit a 7-min half-life to hydrolysis in plasma; PG-EAs are stable

to hydrolysis in serum and plasma indefinitely (37). Interestingly, PG-Gs are rapidly hydrolyzed in rat serum (*e.g.* $t_{1/2}$ for PGE_2-G = 14 s) but are stable indefinitely in cerebrospinal fluid (37).

Biological Effects of PG-Gs

The ability of COX-2 to oxygenate 2-AG and AEA to endoperoxides that are converted to PG-Gs or PG-EAs raises the possibility that this is part of a COX-2-selective signaling pathway. An extensive survey of the biological effects of PG-Gs and PG-EAs is not available, but initial reports are intriguing. PGE_2-G mobilizes Ca^{2+} in RAW264.7 cells at pM to nM concentrations concomitant with a transient elevation of IP_3 levels; Ca^{2+} mobilization is abolished by the IP_3 receptor antagonist TMB-8 (38). Depletion of extracellular Ca^{2+} diminishes but does not eliminate the response, consistent with an initial release of Ca^{2+} from intracellular stores followed by capacitative entry. Depletion of Ca^{2+} from endoplasmic reticulum stores by pretreatment of cells with thapsigargin inhibits the PGE_2-G response. Membrane translocation of protein kinase C is observed along with downstream phosphorylation of ERK and subsequent transcriptional activation dependent on the serum response element. PGE_2-G-dependent Ca^{2+} mobilization and downstream signaling in RAW cells appear to be independent of hydrolysis to PGE_2 (38).

PGE_2-G induces an increase in the frequency of mIPSCs in mouse hippocampal neurons with an EC_{50} of 1.7 μM (39). This contrasts with the effect of its precursor, 2-AG, which, at a concentration of 1 μM, reduces the frequency of mIPSCs. Interestingly, AEA also reduces the frequency of mIPSCs, but PGE_2-EA does not increase the frequency. PGD_2-G, $PGF_{2\alpha}$-G, and PGD_2-EA increase the frequency of mIPSCs, but $PGF_{2\alpha}$-EA does not. The classical PGs PGE_2 and PGD_2 reduce the frequency of mIPSCs, whereas $PGF_{2\alpha}$ has no effect, suggesting that the ability of PG-Gs and PG-EAs to increase the frequency of mIPSCs is not due to hydrolysis to PGs or binding to PG receptors. Direct measurement of the binding of PGE_2-G to cloned and expressed EP receptors reveals that it is at least 2 orders of magnitude less potent than PGE_2 at binding to any of the EP receptors (38).

The increased frequency of mIPSCs observed following treatment with PGE_2-G is not inhibited by a CB_1 receptor antagonist but is inhibited by an IP_3 receptor antagonist and a MAPK inhibitor. The sum of these observations suggests that the increased frequency of mIPSCs is triggered by interactions of PGE_2-G, PGD_2-G, $PGF_{2\alpha}$-G, or PGD_2-EA with novel receptors and that intracellular Ca^{2+} mobilization and MAPK-dependent phosphorylation are involved in the downstream signaling. When hippocampal preparations are treated with the COX-2 inhibitor NS-398, the frequency of mIPSCs decreases, and when they are treated with the COX-2 inducer IL-1β, it increases (39). The increase in mIPSCs triggered by IL-1β treatment is prevented by an IP_3 receptor antagonist and a MAPK inhibitor, suggesting that the increase is due to increased synthesis of endocannabinoid-derived mediators produced by COX-2.

PGE_2-G enhances glutamatergic synaptic transmission in hippocampal neurons as evidenced by an increased frequency of miniature excitatory postsynaptic currents (40). The increase in glutamatergic transmission correlates to enhanced neuronal apoptosis as revealed by caspase-3 cleavage and enhanced TUNEL staining. PGE_2-G signaling occurs through ERK, p38 MAPK, IP_3, and NF-κB pathways. In contrast to PGE_2-G, 2-AG inhibits miniature excitatory postsynaptic currents in hippocampal neurons. The inhibitory effects of 2-AG are inhibited by cannabinoid receptor (CB_1) antagonists, whereas the actions of PGE_2-G are not.

AEA inhibits the LPS/interferon-γ-induced expression of IL-12 family members (IL-12 and IL-23) in microglia/macrophages by reducing the expression of the common subunit, IL-12p40 (41). The COX-2 oxygenation product, PGE_2-EA, exhibits a similar inhibitory effect at both the cellular and molecular levels (*i.e.* transcriptional inhibition through the GA-12 element). The inhibitory effects of AEA in either microglial or RAW264.7 cells are reversed by treatment with the COX-2 inhibitor NS-398, whereas the effects of PGE_2-EA are not. The effects of both AEA and PGE_2-EA are partially reversed by the EP_2 receptor antagonist AH6809, but not by the EP_4 antagonist AH23848B. Thus, the inhibitory effects of PGE_2-EA on IL-12p40 transcription may be partially mediated by interaction with the EP_2 receptor.

Amide derivatives of $PGF_{2\alpha}$ are marketed to lower ocular pressure and for the treatment of glaucoma (42). There has been considerable controversy about whether the amides act as prodrugs for $PGF_{2\alpha}$ or its carboxylic acid analogs following hydrolysis or whether "prostamides" act by binding the FP receptor. Investigations of human ocular tissue have revealed the existence of six splice variants of the FP receptor (43). Immunoprecipitation experiments indicate that a heterodimer is formed between the FP receptor and a variant FP receptor (altFP). Coexpression of the FP and altFP receptors in Epstein-Barr virus nuclear antigen-expressing HEK293 cells dramatically increases the sensitivity of the cells to Ca^{2+} mobilization by prostamides. Besides increasing the sensitivity of the transfected cells to prostamide signaling, the heterodimer is responsible for the induction of a second wave of Ca^{2+} mobilization following prostamide treatment.

The activities summarized above may result from the interaction of PG-Gs or PG-EAs with orphan receptors, classical PG receptors, or heterodimers of PG receptors with splice variants of PG receptors. All of these are members of the G-protein-coupled class of seven-transmembrane cell-surface receptors. Evidence also exists for the activation of the nuclear receptor PPARδ by PGI_2-G. Treatment of human vascular endothelial cells with 2-AG causes PPARδ activation, which down-regulates expression of tissue factor (44). Tissue factor is a procoagulant released by endothelial cells that can lead to clot formation. The extent of PPARδ activation is much greater when cells are treated with 2-AG than when they are treated with AA. The two major metabolites of AA and 2-AG in vascular endothelial cells are PGI_2/PGI_2-G and $PGF_{2\alpha}$/$PGF_{2\alpha}$-G. siRNA knockdown of PGI synthase but not PGF synthase abolishes PPARδ activation by 2-AG. Thus, it appears that human vascular endothelial cells convert 2-AG into PGI_2-G, which activates PPARδ and down-regulates tissue factor expression. 2-AG-dependent PPARδ activation is reduced by the COX-2-selective inhibitor NS-398, but not by the COX-1-selective inhibitor valeryl salicylate. NS-398 increases the production of tissue factor by endothelial cells. The role of tissue factor in promoting clotting sug-

gests that reduction in the levels of PGI_2-G in the vascular endothelium may contribute to the cardiovascular toxicity associated with COX-2 inhibition (45).

Cellular Biosynthesis of PG-Gs

There are limited data on the production of PG-Gs or PG-EAs *in vivo*. PGE_2-EA and PGD_2-EA have been detected in the kidneys and lungs of mice following intravenous injection of AEA (46). The levels of these compounds were much higher after AEA administration to animals bearing a targeted deletion of the gene for fatty-acid amide hydrolase, which rapidly hydrolyzes AEA to AA. $FAAH^{-/-}$ mice also exhibited detectable levels of PGE_2-EA and PGD_2-EA in the liver and small intestine, and $PGF_{2\alpha}$-EA was found in all four tissues. PG-EAs were not detected in animals that had not received exogenous AEA. In contrast, PGE_2-G was detected and rigorously identified in extracts of rat paws from animals that had received no prior treatment (47).

Detailed studies have been reported of the production of PG-Gs in freshly isolated RPMs and the RAW264.7 cell lines (23, 30, 48). They reveal that PG-Gs are formed following release of 2-AG from endogenous stores by treatment with a variety of physiological (LPS and zymosan) and non-physiological (Ca^{2+} ionophore) agonists. The profile of PG-Gs matches that of PGs generated from endogenous AA (for example, PGE_2-G and PGI_2-G from RPMs and PGD_2-G from RAW264.7 cells) (23, 48). Quantification of PG-G and PG biosynthesis indicates that PG-Gs are produced at significantly lower levels than PGs (500–1000-fold) (23, 48). Part of this differential is due to the lower level of 2-AG than AA released by agonist treatment. But other factors may be important in the extent of 2-AG oxygenation. For example, addition of exogenous 2-AG leads to rapid production of AA and PGs as well as PG-Gs (23). In fact, the levels of PGs generated from exogenous 2-AG are 10-fold higher than those of PG-Gs. Thus, 2-AG is rapidly hydrolyzed to AA in both RPMs and RAW cells. Another factor appears to be the transient nature of COX-2 action. In RPMs, integration of PG-G formation indicates that COX-2 may be active only for a short time (~20–40 min), whereas COX-1 appears to be active for several hours (23). This biases the pattern of oxygenation products in favor of PGs because AA is an excellent substrate for COX-1 and COX-2, whereas 2-AG is a 10–20-fold better substrate for COX-2. Finally, there may be major differences in the sites of release of AA and 2-AG relative to the localization of COX-2 as well as the efficiencies of delivery to the enzyme. It will be interesting to determine whether similar complexities exist in 2-AG release and PG-G formation in other cell types (*e.g.* neurons and vascular endothelial cells).

Definition of the pathways of release of 2-AG and the precursor pools from which it is derived is equally challenging. Literature precedent suggests that 2-AG is derived from hydrolysis of DAG (49, 50). Whether DAGs are generated by phospholipases C or phospholipases D followed by phosphatase action is uncertain. Most of the small molecule inhibitors that are available are not selective and inhibit multiple pathways of phospholipid hydrolysis and, in some cases, fatty acid oxygenation (51).[3]

[3] A. Vila and L. J. Marnett, unpublished data.

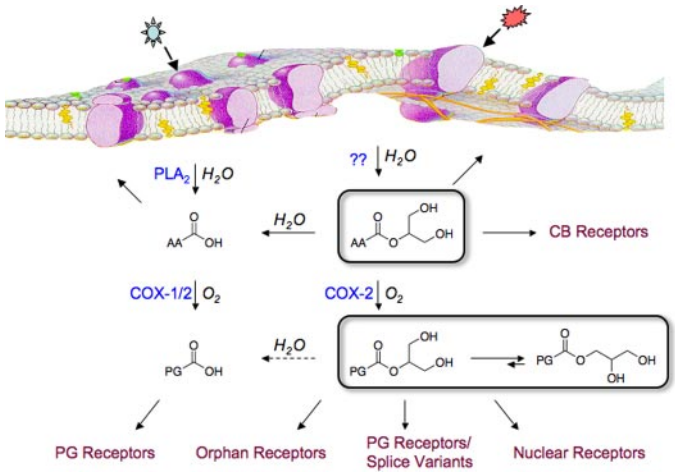

FIGURE 3. **Agonist-dependent oxygenation of AA and 2-AG by the COX enzymes.** The interaction of an agonist with its cell-surface receptor leads to the release of free AA from membrane phospholipids via the action of PLA_2. The free AA is subject to oxygenation by both COX isoforms, leading to the formation of PGs, or it may be reincorporated into the membrane. 2-AG is formed via a PLC- or PLD-mediated pathway. It may be oxygenated by COX-2, leading to the formation of PG-Gs. Alternatively, it may be hydrolyzed to free AA or reincorporated into the membrane. Hydrolysis of PG-Gs leads to their conversion to PGs. PGs formed from hydrolysis of PG-Gs are indistinguishable from PGs formed by direct oxygenation of AA. 2-PG-Gs can isomerize to 1-PG-Gs with a half-life of 4–10 min. Many of the same elements of this scheme may pertain to AEA as well as 2-AG.

siRNA knockdown reagents are being developed for use in RAW264.7 cells, which may provide definitive approaches to defining the involvement of specific phospholipases and DAG lipases.

Massively parallel lipid profiling is being employed to define the phospholipid pools that are mobilized following cell stimulation (52, 53). For example, it is possible to quantify separately each DAG species and to monitor its turnover following LPS, zymosan, or LPS/zymosan treatment. By a combination of siRNA techniques and lipid profiling, it should be possible to precisely define the pools of lipids that lead to PG-Gs and to catalog their natural history following cell stimulation.

Conclusion

The discovery of an inducible isoform of COX that exhibits increased expression in inflammatory tissue immediately led to the hypothesis that this isoform is primarily responsible for the well known contribution of COX to the inflammatory response. The anti-inflammatory efficacy of COX-2-selective inhibitors supports this hypothesis. However, the more recent discovery of the cardiovascular toxicity of these inhibitors clearly illustrates that COX-2 mediates an array of additional physiological processes (54). It is possible that the distinct roles of COX-1 and COX-2 depend entirely upon their differential patterns of expression. However, the recent studies with COX-1 knock-in mice suggest that there are subtle differences in enzyme function that prevent one isoform from fully substituting for the other. The ease of hydroperoxide activation of COX-2 compared with COX-1 may provide the basis for these differences. However, the ability of COX-2 to oxygenate neutral AA derivatives leads to an intriguing alternative hypothesis as outlined in Fig. 3. Free AA resulting from phospholipid hydrolysis by cytosolic PLA_2 is a substrate for both COX isoforms. In con-

trast, 2-AG formed via a PLC- or PLD-mediated pathway is a selective substrate for COX-2. Endocannabinoid oxygenation may give rise to COX-2-specific metabolites with a unique repertoire of physiological activities mediated by orphan G-protein-coupled receptors, heterodimers of eicosanoid receptors and their splice variants, or nuclear lipid receptors. Alternatively, this function of COX-2 may play a role in modulating endocannabinoid tone (55). Available data support both of these conjectures, including demonstrations of the biological activity of PGE_2-G, PG-G biosynthesis by intact cells, and a role for COX-2 activity in regulating endocannabinoid-dependent neuronal processes. As indicated in Fig. 3, 2-AG is subject to hydrolysis, producing AA, which may be oxygenated to form PGs. Hydrolysis of PG-Gs also produces PGs, which are indistinguishable from those produced from direct AA oxygenation. Consequently, determination of the importance of COX-2-dependent endocannabinoid oxygenation *in vivo* presents a significant challenge. Nevertheless, a full understanding of the potential role of this pathway may lead to important insights into the function of COX-2 in human health and disease.

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